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CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND CLONING OF ITS GENE

ANNUAL REPORT

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The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an *in vitro* system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs. We have raised polyclonal antibodies to crotoxin and its subunits from *Crotalus durissus terrificus*, Mojave toxin from *Crotalus scutulatus scutulatus*, concolor toxin from *Crotalus viridis concolor*, and monoclonal antibodies to crotoxin. We have used these to search for cross-reactive, homologous proteins in other venoms and examine their toxin neutralizing ability. The amino acid sequences of both crotoxin subunits were determined as a prelude to cloning. Based on this sequence information, we synthesized deoxyoligonucleotide probes complementary to the crotoxin gene to identify positive clones. (See Con't

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19. Abstract (Con't)

Spectral measurements have been made on crotoxin, its subunits, and related toxins using ultraviolet, circular dichroism, and fluoresence spectroscopy, to provide information on its secondary structure. We have also initiated a collaboration with an x-ray crystallography group and are making progress toward solving the crystal structure of Mojave toxin. Cross-linkers have been used to chemically link crotoxin subunits to determine whether they need to dissociate for expression of toxicity.

Results with the polyclonal antibodies indicate that crotalid neurotoxins can be detected and neutralized by polyclonal antibodies raised against any intact toxin or basic subunit in this group of homologous toxins. Four different monoclonal antibodies have been purified and partially characterized, with one of these showing potent crotoxin neutralizing activity. This neutralizing monoclonal antibody is being used to raise anti-idiotype antibodies which will be examined for their potential as anti-idiotype vaccines. The complete amino acid sequence of the basic subunit and two of the three acidic subunit chains of crotoxin from the venom of C.d. terrificus has been determined. Sequence comparison data suggest that the non-toxic, acidic subunit was derived from an non-toxic, homodimeric, crotalid phospholipase A_2. We have examined a number of other crotalid venoms for the presence of crotoxin homologs and found Crotalus vegrandis to contain a venom component that is structurally and antigenically similar to crotoxin. X-ray diffraction studies on Mojave toxin and the spectral studies on crotoxin, its subunits, and homologous toxins are progressing well and should enhance our structural understanding of the rattlesnake presynaptic neurotoxins. Cross-linking experiments now underway should enable us to determine whether crotoxin subunit dissociation is essential for neurotoxicity, and may provide insight into its mechanism of action.

SUMMARY

The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs. We have raised polyclonal antibodies to crotoxin and its subunits from Crotalus durissus terrificus, Mojave toxin from Crotalus scutulatus scutulatus, concolor toxin from Crotalus viridis concolor, and monoclonal antibodies to crotoxin. We have used these to search for cross-reactive, homologous proteins in other venoms and examine their toxin neutralizing ability. The amino acid sequences of both crotoxin subunits were determined, as a prelude to cloning. Based on this sequence information, we synthesized deoxyoligonucleotide probes complementary to the crotoxin gene to identify positive clones. Spectral measurements have been made on crotoxin, its subunits, and related toxins using ultraviolet, circular dichroism, and fluoresence spectroscopy, to provide information on its secondary structure. We have also initiated a collaboration with an x-ray crystallography group and are making progress toward solving the crystal structure of Mojave toxin. Cross-linkers have been used to chemically link crotoxin subunits to determine whether they need to dissociate for expression of toxicity.

Results with the polyclonal antibodies indicate that crotalid neurotoxins can be detected and neutralized by polyclonal antibodies raised against any intact toxin or basic subunit in this group of homologous toxins. Four different monoclonal antibodies have been purified and partially characterized, with one of these showing potent crotoxin neutralizing activity. This neutralizing monoclonal antibody is being used to raise anti-idiotype antibodies which will be examined for their potential as anti-idiotype vaccines. The complete amino acid sequence of the basic subunit and two of the three acidic subunit chains of crotoxin from the venom of C. d. terrificus has been determined. Sequence comparison data suggest that the non-toxic, acidic subunit was derived from an non-toxic, homodimeric, crotalid phospholipase A_2. We have examined a number of other crotalid venoms for the presence of crotoxin homologs and found Crotalus vegrandis to contain a venom component that is structurally and antigenically similar to crotoxin. X-ray diffraction studies on Mojave toxin and the spectral studies on crotoxin, its subunits, and homologous toxins are progressing well and should enhance our structural understanding of the rattlesnake presynaptic neurotoxins. Cross-linking experiments now underway should enable us to determine whether crotoxin subunit dissociation is essential for neurotoxicity, and may provide insight into its mechanism of action.

FOREWORD

Citations of commerical organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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STATEMENT OF PROBLEM

This contract supports studies to (1) examine the structural properties of crotoxin and closely related homologous, rattlesnake neurotoxins; (2) examine in an in vitro system, the mechanism of action of crotoxin as a model of a class of snake venom presynaptic neurotoxins; and (3) clone the crotoxin gene as a first-step in generating a non-toxic but immunoreactive crotoxin analog.

BACKGROUND

The discovery of crotoxin, a potent, enzymatic neurotoxin from the venom of the South American rattlesnake (Crotalus durissus terrificus) by Slotta and Fraenkel-Conrat (1) marked the beginning of modern-day snake venom research. Crotoxin is a heterodimeric protein, consisting of a moderately toxic basic phospholipase A_2 and a acidic, non-toxic subunit composed of three small proteins (2). The acidic subunit is required for full toxicity, but has no other identified function. Evidence gradually accumulated suggesting similarity between crotoxin, Mojave toxin from Crotalus s. scutulatus, concolor toxin from Crotalus viridis concolor, and vegrandis toxin from Crotalus vegrandis, although disconcerting differences persisted (see ref. 3 and 4 for review). From our early investigations (5), we were reasonably certain that the above four toxins were similar structurally and functionally. We wanted to extend these studies and prepared rabbit antiserum against crotoxin, concolor toxin, and Mojave toxin, as well as against the acidic and basic subunits of crotoxin. These antisera were used to examine the antigenic relatedness of purified crotalid toxins and subunits by both double immunodiffusion and ELISA. We also determined the relative efficacy of antisera raised against the subunits and intact complex of crotoxin in neutralizing the toxicity of crotoxin and related toxins in mice (6). At the same time we were interested in preparing monoclonal antibodies to crotoxin. These would provide us with additional tools to probe crotoxin structure and if we were fortunate enough to generate a neutralizing monoclonal antibody, might provide for either the development of an anti-idiotype vaccine, or the identity of a neutralizing antigenic site on crotoxin (7).

Crotoxin has been the most extensively studied of the rattlesnake neurotoxins and will serve as the basis of comparison for all others. Its characterization then, should be as complete as possible. When this work was started, the amino acid sequence for the basic subunit protein from crotoxin was largely determined, with the exception of the Asp/Asn and Glu/Gln residues, which could not be differentiated (8). This partial sequence facilitated the chemical and enzymatic fragmentation studies in our laboratory. It was necessary that we distinguish between Glu/Gln residues since the best region to which to prepare a synthetic cDNA probe, based on the published sequence, showed three Glx residues (residues 82-84, Fig. 10). This region has little homology with known sequences in non-toxic phospholipases. We have now completed the amino acid sequence of the basic subunit (9) and two of the three acidic subunit chains (2). We have all but ten amino-terminal residues of the third chain sequenced.

While we have sequenced the acidic and basic subunit protein chains of crotoxin, the unavailability of C. d. terrificus necessitated using a close congener, C. vegrandis, a durissus derivative found in the center of C. d. terrificus range, for cloning studies. It has been shown to have a major venom component that is structurally and antigenically similar to crctoxin (6,7). We now have one mature and 21young C. vegrandis in our snake facility, which we obtained from Mr. Hugh Quinn, curator of reptiles at the Houston zoo. These will be available for future work. We have also just located five C. d. terrificus and should be adding these to our collection

within the next two weeks. These will be used for future cloning studies.

Hanley (10) carried out structural studies on crotoxin and its subunits, employin fluorescence and circular dichroism (CD) measurements. He reported that the complex contained an increased proportion of ordered secondary structure (particularly ß-sheet), compared to the individual subunits. Hanley could not distinguish between conformation changes within the subunits upon interaction or the creation of ordered structure at the subunit binding interface. Tu and coworkers (11) used Raman spectroscopy and CD to examine the peptide backbone conformations of native and reassociated Mojave toxin and its subunits. They concluded that when the subunits reassociated, the peptide backbone, disulfide bond conformation, and the microenvironment of Tyr side chains were largely unchanged. Further, the native conformation, as well as the individual subunits, consisted mainly of α-helix structure. It is difficult to reconcile the results of these two groups if we assume that crotoxin and Mojave toxin are structurally similar. Physical studies are in progress on crotoxin, Mojave toxin, concolor toxin, and vegrandis toxin and their subunits, that should resolve these differences once and for all.

Previous work by Hendon and Tu (12) was designed to examine whether dissociation was essential for neurotoxicity. Dimethyl suberimidate (DMS), was used to irreversibly bind the two subunits. Their data suggest that they introduced an average of three crosslinks per complex. At least one of these must have been between subunits because recovered complex could not be dissociated in 6M urea. Sequence analyses indicate the presence of 10 Lys residues (the most likely reactant) in the basic subunit and one each in the A-chain and B-chain of the acidic subunit (2). The DMS-crotoxin had comparable levels of phospholipase A_2 activity to that of unmodified crotoxin (21 µmol/min-mg). LD_{50} values in mice increased from 0.06 µg/g to >1.5 µg/g. Retention of phospholipase activity and loss of neurotoxicity in the cross-linked complex was interpreted to reflect "interference beteween the cross-linked complex and the target site." Recent results from two different groups indicate that loss of neurotoxicity may have resulted from modification of the ε-amino group of Lys and not necessarily cross-linking. Using chemical derivatization techniques, Rosenberg's group (13) observed that Lys or Arg group modification in basic phospholipase A_2 enzymes, frequently resuits in greater loss of pharmacological than of enzymatic activity. They note that modification of these basic amino acids may alter the protein's stability, distribution, or tissue binding ability. Jeng and Fraenkel-Conrat (14) also observed inactivation of neurotoxicity in crotoxin upon acetylaton of amino groups and suggested that the inactivation was due to a "discrete change in the conformation of the molecule induced by the loss of positive charges." Hendon and Tu also used DEAE-cellulose column chromatography to separate cross-linked and unreacted toxin components in phosphate-buffered urea. Their neurotoxicity loss may have resulted, in part, from carbamylations during chromatography resulting from spontaneous breakdown of urea generating cyanate which reacts with amino groups. Their unreacted controls were not cycled over urea columns. We are repeating Hendon and Tu's cross-linking experiments with the appropriate controls, in addition to examining two additional crotoxin subunit cross-linkers.

Little is known about the biosynthesis of crotoxin or for that matter any venom proteins found in reptiles. This proposed cloning study should permit us to answer fundamental questions concerning crotoxin and related crotalid neurotoxins. It will also provide a cloned crotoxin gene that will permit manipulation and selective alteration. Future oligonucleotide-directed site-specific mutagenesis promises to be an extremely powerful tool with which to probe encoded proteins. We have made two different attempts to clone the crotoxin gene, but both were unsuccessful. We are currently working to improve our efficiency of mRNA extraction from

venom glands. Once this is completed we will have another try at cloning.

APPROACH TO THE PROBLEM

We have used a multi-faceted approach in our study of crotoxin, which has included tools from the disciplines of biochemistry, chemistry, and immunology. Crotoxin from C. d. terrificus, its subunits and homologous toxins from C. s. scutulatus and C. v. concolor have been isolated and purified by a combination of gel filtration and ion exchange column chromatography. These purified materials were then used to generate polyclonal antibodies in rabbits and monoclonal antibodies in mice. Neutralization properties of these antibodies have been examined using purified toxins, crude venoms, as well as determiniation of their cross-reactivity with a variety of other proteins.

One monoclonal antibody line is a potent neutralizing antibody of crotoxin. It has been purified and used to raise anti-idiotype monoclonal antibodies, in collaboration with Dr. John L. Middlebrook, Pathology Division, USAMRIID. These anti-idiotype monoclonal antibodies will be examined for possible use as an anti-idiotype vaccine. We showed earlier that Mojave toxin from C. s. scutulatus and concolor toxin from C. v. concolor were functionally and structurally very similar to crotoxin (5). In a recently submitted manuscript (15), we demonstrated homology of yet another neurotoxin from the venom of C. vegrandis. Because of the availability of C. vegrandis, we have used venom glands from these animals for our cloning studies. We have examined the venom of several additional rattlesnakes for the presence of a crotoxin-like toxin, including Crotalus viridis lutosus. After a preliminary fractionation and characterization (16), we found none in this venom.

Purified subunits of crotoxin have been used for sequencing as well as for examining individual subunit spectral properties using fluorescence and circular dichroism. These spectral determinations have been run in parallel with additional measurements on several intact, homologous toxins. Results will be combined with those currently in progress using deconvolution FTIR, in an effort to detect structural changes that result from subunit reassociation. Cross-linking of the acidic and basic subunits of crotoxin have been examined using the water soluble carbodiimide EDC [1-ethyl-3(3-dimethylaminopropyl)carbodiimide] and two bifunctional cross-linkers EGS [ethylene glycolbis(succinimidylsuccinate)] and DST (disuccinimidyl tartarate). Products of these reactions are routinely assayed by SDS-PAGE and cross-linked material is isolated by column chromatography in the presence of urea. Toxicity of the cross-linked toxins are determined in mice.

Samples of purified Mojave toxin have been provided to the laboratory of Dr. Keith Ward, Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D.C. for crystallization studies. His laboratory has been successful in obtaining crystals suitable for x-ray diffraction studies. Detailed x-ray structural analysis should provide valuable insight into rattlesnake presynaptic neurotoxin structure.

We have attempted two different clonings of the "crotoxin" gene in collaboration with Dr. Leonard Smith, Pathology Division, USAMRIID. In both instances C. vegrandis were milked and three days later their venom glands were surgically removed from anesthetized snakes. These were frozen quickly in liquid nitrogen and subsequently extracted for RNA. cDNA was prepared from mRNA removed from a poly-dT column and cloned into a puc9 plasmid. Synthetic deoxynucleotide probes suggested positive clones for crotoxin genes, but subsequent sequencing of recovered DNA did not confirm this.

An in vitro system for the examination of the biological effects of presynaptic neurotoxins is sorely needed. We recently completed an extensive library search and collections of manuscripts describing the preperation and properities of synaptosomes from the electric organ of the electric ray (Torpedo and Narcine). This system appears to be one of the best currently available. We are continuing our evaluation of this system to determine whether it will satisfy our in vitro assay requirements.

RESULTS

I. POLYCLONAL ANTIBODIES. As an extension of our earlier comparative studies, we prepared rabbit antisera against crotoxin, concolor toxin (C. v. concolor) and Mojave toxin (C. s. scutulatus), as well as against the acidic and basic subunits of crotoxin. These antisera were used to examine the antigenic relatedness of purified crotalid toxins and subunits by both double immunodiffusion and ELISA. We have also determined the relative efficacy of antisera raised against the subunits and intact complex of crotoxin in neutralizing the toxicity of crotoxin and related toxins in mice. (See ref. 6 for experimental details).

IMMUNODIFFUSION. In double immunodiffusion studies, antisera raised against intact crotoxin gave a single major precipitin line against homologous antigen at concentrations as low as 0.064 mg/ml (Fig. 1A). Antisera against intact concolor and Mojave toxin showed similar levels of sensitivity against their homologous antigens. This same crotoxin antiserum produced lines of identity when reacted with intact crotoxin, concolor toxin, and Mojave toxin (Fig. 1B). A lighter band closer to the center well is also evident in Figs. 1A and 1B. An earlier report noted that reconstituted crotoxin antiserum formed a line of identity against intact crotoxin and Mojave toxin (17). No preimmune sera produced precipitin lines against any antigens.

Using usual immunodiffusion procedures, no bands were observed with the above antisera and either the basic subunit alone or the acidic subunit alone of the three toxins. Earlier workers reported the absence of precipitin lines between antiserum raised to either crotoxin or its basic subunit and individual crotoxin subunits in double immunodiffusion plates (17,18). However, when 0.1% sodium dodecyl sulfate and 1% Triton X-100 were incorporated in the agar, lines of identity were observed for the basic subunits of crotoxin, concolor, and Mojave toxin against all three antisera raised against these intact toxins (Figs. 1C, 1D, and 1E). There is complete fusion of precipitin lines, with no spurs, in these figures, indicating identity between all antigens with all antisera. Detergents did not interfere with precipitation of the antibody-antigen complex, but did permit 'sticky' basic subunits to diffuse out of their wells. Protein staining revealed that in the absence of detergents, basic subunits remained adsorbed to the agar at the well walls. While addition of detergents did permit formation of precipitin lines between basic subunits and reaction antisera, this was not the case with acidic subunits. No precipitin lines were observed with the acidic subunit with any of the antisera under a variety of conditions. This included addition of detergents or polyethylene glycol 6000, variation of the pH from 6 to 8, and increasing the acidic subunit concentration up to 2 mg/ml.

Antisera reaised against the basic subunit of crotoxin also formed precipitin lines of identity with basic subunits of all three toxins (Fig. 1F). The strong precipitin lines shown by the three basic subunits against intact toxin antisera (Figs. 1C, 1D, and 1E) are in stark contrast to the weak lines generated when the basic subunits were tested against crotoxin acidic subunit antiserum. Figure 1F also shows the problem of bubble formation that occurred frequently between the agar and plastic plate surface in the presence of detergents. Finally, antisera raised against subunits of crotoxin were able to form precipitin lines with intact

toxins, as shown against crotoxin in Fig. 1G. Similar patterns were observed against concolor and Mojave toxin.

ELISA. An ELISA method was used to monitor antibody titer in various antisera and for evaluating cross-reactivities. Figure 2 illustrates a typical dilution experiment used to determine the response of various antigens to intact crotoxin antiserum. This sensitive detection method gave readings above background levels at antiserum dilutions of over $1:10^{-6}$, as may be seen with crotoxin antiserum and its reaction with homologous toxin, its basic subunit and the closely related conclolor and Mojave toxins. Antiserum against intact crotoxin was less reactive with the acidic subunit than with either the basic subunit or intact toxins (Fig. 2). Results from a number of such experiments are summarized in Table 1. Antisera against intact crotoxin, concolor, and Mojave toxin exhibited strong homologous and heterologous reactivity. Crotoxin basic subunit antiserum also reacted well with homologous antigen and intact toxins, but gave a weaker reaction with the acidic subunit of crotoxin. While the antiserum against the acidic subunit of crotoxin did not form precipitin lines against its antigen on Ouchterlony plates, a positive ELISA was obtained, although at somewhat reduced levels. Crude venoms from C. d. terrificus and C. s. scutulatus reacted strongly with antisera against crotoxin and its subunits. Purified phospholipase A_2 from C. atrox and C. adamanteus showed weak cross-reactivity with these same antisera, while cross-reactivity with ß-bungarotoxin was very slight. Neurophysin and phospholipase A_2 from N. n. atra and honey bee (A. mellifera) did not react.

TOXIN NEUTRALIZATIONS. Intravenous LD_{50} values for crotoxin, concolor toxin, and Mojave toxin in mice were determined to be 0.056, 0.062, and 0.042 µg/g, respectively. Figure 3 shows that antisera raised against the basic subunit and intact crotoxin were both quite effective in neutralizing crotoxin. Antisera against intact crotoxin neutralized about 330 LD_{50} of crotoxin per ml serum, wheras the basic subunit antiserum neutralized about 460 LD_{50}. Antisera against the acidic subunit showed relatively poor neutralizing ability against crotoxin, with the amount of antisera required to prevent death being ten times that for antisera against intact toxin. When an equivalent LD_{50} dose of either concolor toxin or Mojave toxin was used in place of crotoxin and incubated with antisera against intact crotoxin, neutralization midpoints were similar to those observed for crotoxin. Total dosages for crotoxin, concolor toxin, and Mojave toxin were 0.20, 0.22, and 0.15 µg/g, respectively.

Antigen and antibody were injected separately (i.v.) at various times into mice. This was done to examine the capability of antisera prepared against intact crotoxin or its basic subunit to neutralize crotoxin in vivo. Results showed that antisera, in order to be even partially efficacious, must be injected no more than 1-2 min after toxin injection (Table 2). If antiserum was injected before the toxin, complete protection of the mice was observed. Alternatively, if crotoxin was injected i.m., neutralization was considerably more effective (Table 3); we observed good protection if antisera were administered within 30 min. Some protection was provided if antisera were injected 2 hr after toxin inoculation.

II. MONOCLONAL ANTIBODIES. PURIFICATION. Ascites fluids containing monoclonal antibodies identified as 1, 2, 5, and 11 were derived from hybridomas generated from a mouse immunized with the basic subunit of crotoxin; antibody 6 was derived from an intact crotoxin immunized mouse. All fluids were assayed for their crotoxin neutralizing ability. No protection was found when up to 160 µl of crude ascites fluid was pre-incubated with 20µg

crotoxin/ml for lines 2, 5, 6, and 11. Line 1 demonstrated potent crotoxin neutralizing ability with 1460 LD_{50}'s of crotoxin neutralized per ml of ascites fluid. For unknown reasons all cell lines derived from the mouse immunized with intact crotoxin (antibody 6) did not produce antibodies when given as ascites tumors. All mouse monoclonal antibodies typed as IgG_1 subclass with kappa light chains when assayed in ascites fluid and also following purification.

Immunoglobulins were isolated from ascites fluid by a combination of ammonium sulfate precipitation and protein A-Sepharose column chromatography. Elution profiles from lines 1, 5, and 11 are shown in Figure 4. The profile from line 2 fluid was essentially identical to line1. Under the conditions used, the first 280 nm absorbing peak was devoid of reactive immunoglobulin. While all immunoglobulins were typed as IgG_1 subclass, they all behaved somewhat differently on protein-A columns. When fluids from lines 1 and 2 were dialyzed vs 0.1 M sodium phosphate buffer (pH 8.1) and loaded onto a column equilibrated with 0.1 M sodium phosphate buffer (pH 8.0) at room temperature, the monoclonal antibody was initially retarded, but did slowly bled off until eluted with 0.1 M sodium citrate (pH 6.0). Antibodies from lines 5 and 11 bound to protein A more tightly and were not released to any appreciable extent until the pH was lowered (Figure 4). No immunoglobulin peak was found for sample 6, consistent with the apparent lack of immunoglobulin in the ascites as determined by SDS-PAGE (not shown).

SDS-PAGE on 7.5% gels of various ascites fluid fractions, revealed effective purification of the monoclonal antibodies by the protein A column (Figure 5A). This was confirmed by chromatography of the purified monoclonal antibodies on a high-performance cation exchange column, which demonstrated a single, symmetrical peak for each sample (not shown). Reduction of the purified monoclonal antibodies and SDS-PAGE on 15% gels is shown in Figure 5B.

In double immunodiffusion studies carried out essentially as described (6), mixing of ascites fluid from monoclonal lines 1,2, and 5 gave a weak, broad precipitin band against intact crotoxin (data not shown).

Purified monoclonal antibodies were examined for their reactivity in several ways. First, antibodies 1, 2, and 5 were examined by ELISA (using immobilized antigens) for their reactivity with six different lots of crude C. d. terrificus venom, as well as the purified crotoxins, provided by three different suppliers (Sigma, Miami Serpentarium, and the Butantan Institute, Sao Paulo, Brazil). In addition, we examined the cross-reactivity of three related purified toxins; Mojave toxin, concolor toxin, and vegrandis toxin from C. s. scutulatus, C. v. concolor, and C. vegrandis, respectively. Non-toxic phospholipases A_2 from C. atrox and C. adamanteus were also examined as antigens. All monoclonals reacted with each venom and purified toxin samples, except antibodies 1 and 5, which reacted weakly with concolor toxin. Monoclonal 1 was also less efficient in neutralizing concolor toxin under conditions where it was effective against crotoxin (see later). None of the monoclonal samples reacted with the phospholipase A_2 from C. atrox and C. adamanteus.

We are currently using a sandwich displacement assay, where the solid phase is coated with a monoclonal antibody, and serial dilutions of various proteins are tested for competitive inhibition of binding of biotinylated-crotoxin. These assays are in progress for the four purified monoclonal antibodies.

NEUTRALIZATION OF CROTOXIN AND CRUDE C. D. TERRIFICUS VENOM. Purified monoclonal antibody 1, neutralized 14.7 µg of crotoxin with 61 µg of protein. This translates to 0.639 nmol crotoxin neutralized by 0.407 nmols of the monoclonal for a 1.57 molar ratio. Thirty three hundred µg of Wyeth crotalid polyvalent horse antivenin was required to neutralize

the same amount of crotoxin. Monoclonal antibody 1, was therefore about 54X more effective in neutralizing purified crotoxin on a weight or molar basis than this lot of commerical polyvalent horse antivenin.

Crude C. d. terrificus venom (lot CJ 5BZ and CD14JXZ, Miami Serpentarium) had an LD_{50} of 0.075 µg/g in male mice. We estimated that 50% of the protein in these samples was crotoxin. The ability of monoclonal antibody 1 to neutralize the toxicity of crude venom when used at an antibody to crotoxin molar ratio of 2:1 was based on the above estimate. This calculates to be about a 3.2-fold excess of the monoclonal required for complete neutralization. Surprising, the monoclonal antibody that was potent at neutralizing purified crotoxin was not effective against the lethality of the crude venom (Table 4). Wyeth's polyvalent crotalid antivenin, on the other hand, was quite effective against crude venom. When used at a concentration equivalent to that amount of monoclonal antibody used to neutralize purified crotoxin, no deaths were observed (Table 5).

The experiment providing the results shown in Table 5 was continued, using increasing amounts of crude venom and constant amounts of Wyeth antivenin. Results are shown in Table 6. At higher concentrations of crude venom, it was possible that the crotoxin neutralization capacity of the polyvalent antivenin was exceeded. If so, supplementation of the polyvalent antivenin with the monoclonal antibody should permit a higher tolerance of crude venom. This was indeed the case as shown in Table 7. These results indicate that some venom component(s) other than crotoxin is responsible for the animal's death when monoclonal antibody is used as a neutralizing antibody with crude venom. This component(s) is antigenic, since Wyeth antivenin effectively neutralizes the component(s). The finding that the monoclonal antibody can enhance the effectiveness of Wyeth antivenin in neutralizing crude C. d. terrificus venom suggests that crotoxin in crude venom is available to the antibody.

CROSS-NEUTRALIZATIONS. Sandwich ELISAs indicated that Mojave toxin cross-reacted more strongly with the neutralizing monoclonal antibody 1 than did concolor toxin. If the pattern also held in neutralization, then Mojave toxin should be more effectively neutralized than concolor toxin with the antibody.

Neutralization of Mojave toxin was carried out using crude venom of C. s. scutulatus having an LD_{50} of 0.10 µg/g in male mice. It was estimated that about 50% of the total venom protein represented Mojave toxin. After 24 hours, all mice appeared normal with no indication of toxicity (Table 8). These results indicate that Mojave toxin is neutralized by monoclonal antibody 1. Apparently, at three times the LD_{50} value of the crude venom, other venom components are still below their LD_{50} value. This observation was in contrast with crude venom from C. d. terrificus, where injection of about two times the LD_{50} of the crude venom generally resulted in rapid mouse death (in less than 5 minutes), in the absence or presence of a two-fold molar excess of monoclonal antibody 1 (Table 4).

Effectiveness of monoclonal antibody 1 at neutralizing concolor toxin was then examined. Female mice were injected with constant amounts of purified concolor toxin (0.283 µg/g or 12.3 nmols toxin /g), equivalent to 3.8 times LD_{50} of concolor toxin (LD_{50} = 0.075 µg/g in female mice). This toxin was preincubated with varying concentrations of monoclonal antibody 1 up to a 2 to 1 molar ratio of antibody to toxin. All mice died within 24 hours, although mice not receiving antibody had a shorter time-to-death, suggesting partial protection. This partial protection was also seen when lower concentrations of concolor toxin were used, and the molar ratio of antibody:toxin was maintained at 2 (Table 9). This partial cross-reaction of concolor

toxin with monoclonal antibody 1, is consistent with the sandwich ELISA results, where concolor toxin interferes with biotinylated-crotoxin binding to the monoclonal antibody, but less effectively than intact crotoxin, Mojave toxin, and vegrandis toxin. For expeimental details see ref. 7.

III. CROTOXIN HOMOLOGS IN OTHER RATTLESNAKE VENOMS. (A) CROTALUS VEGRANDIS--CHROMATOGRAPHY, TOXICITY, AND IMMUNOLOGICAL ASSAYS. Crude venom from C. vegrandis was initially fractionated by gel filtration, which yielded a peak containing about 42% of the recovered protein (Fig. 6). Crotoxin and related crotoxin presynaptic neurotoxins elute in the same position. Subsequent fractionation on an anion exchange column (Mono Q) of this pooled peak yielded three discrete peaks (Fig. 7). When pooled and rerun on anion exchange, peaks 2 and 3 underwent extensive dissociation. These same two peaks showed LD_{50} values similar to that of crotoxin (≈0.05 µg/g in mice), wheras peak 1 had a LD_{50} >0.08 µg/g. All three peaks were run in double immunodiffusion gels with antisera raised against intact crotoxin. This antiserum produced lines of identity with crotoxin and with all three neurotoxin peaks (Fig. 8). In results not shown, all three peaks recovered from Mono Q were used as plate coating antigens (each at 1 µg/ml), along with crotoxin, and then reacted with serially disuted rabbit antisera raised against crotoxin. All four rows of well coated with the four different antigens reacted in a similar manner, decreasing in absorbance in parallel at greater antiserum dilutions. When intact crotoxin was replaced with its basic and acidic subunits in two subsequent assays, rabbit antisera raised against the crotoxin subunits cross-reacted equally with the three vegrandis isotoxins. That is, absorbances diminished proportionally to antiserum dilution, and the diminution of absorbance for the three vegrandis isotoxins paralled that of the crotoxin subunits. This indicated that the vegrandis isotoxins contain moieties that are immunologically identical to the subunits of crotoxin.

SDS-PAGE. SDS-PAGE in the absence of sulfhydryl reducing agents dissociates the non-covalently associated subunits of crotoxin and resolves the two subunits(5). The three neurotoxin peaks from C. vegrandis venom (Fig. 7), dissociated into subunits with mobilities identical to the basic and acidic subunits of crotoxin. There appear to be at least two different forms of the acidic subunit in the three isotoxins from C. vegrandis. These co-migrate with the two different forms of acidic subunit seen in crotoxin (5) . It is not clear why the acidic subunits of crotoxin differ in their mobility in the presence of SDS, since both should have similar charge/mass ratios. When reducing agent is added to the samples before electrophoresis, all basic subunits migrate with identical mobilities. Acidic subunits disappear because of disulfide bond reduction and dissociation of the three small peptides.

In addition to examining the chromatographic profiles of individual and pooled, adult venoms from C. vegrandis, we fractionated eight individual crude venoms from juvenile snakes on Mono Q. In all cases we observed multiple components that eluted as had the neurotoxin isomers in adult venoms. Aliquots of these peaks from the juvenile venoms also reacted positively in double antibody sandwich ELISA using a combination of rabbit polyclonal and mouse monoclonal antibodies (Fig. 9). A peak eluting in the void volume, comprised at least partially of the basic subunit of vegrandis toxin, also reacted strongly in the ELISA.

(B) CROTALUS VIRIDIS LUTOSUS. Crude venom from Crotalus viridis lutosus was fractionated over the gel filtration medium Sephacryl S-200. Each fraction was examined by SDS-PAGE and selected fractions by reverse-phase liquid chromatography. Based on these profiles, enzymatic assays, toxicity assays in mice, and immunoassays, we found no evidence for

the presence of a crotoxin homolog in the venom (16). This is in contrast with the conspecific taxon, Crotalus viridis concolor (5), held by Klauber to be a stunted offshoot of C. v. lutosus. Experimental details for work on C. vegrandis and C. v. lutosus may be seen in ref. 15 and 16.

IV. SEQUENCING OF CROTOXIN. (A) BASIC SUBUNIT. The complete sequence of the basic subunit of crotoxin was obtained by a combination of direct sequencing from the amino terminus of the protein and by sequencing cleavage fragments obtained after digestion with the specific endoproteases Arg-C and Lys-C, or after incubation with cyanogen bromide (Fig. 10). The latter method produced quantitative cleavage at both Met residues.

Arg-C and Lys-C both showed variable specificity for their respective target residues and for one unanticipated site. Arg-C consistently cleaved the bond between Tyr-24 and Gly-25 with high efficiency. It hydrolyzed the Arg-Pro bond between positions 35 and 36 poorly, as would be expected, and it repeatedly showed no activity against Arg-42, which is flanked on the carboxyl side by two Cys residues. It may be that the two methylcarboxyl groups attached to these residues during carboxymethylation interfere with enzyme binding. Arg-C cleaved with high yields at Arg-14, Arg-37, Arg-65, Arg-90, Arg-97, and Arg-98 (Fig. 10). No attempt was made to retrieve the two C-terminal Arg-C peptides because that region had already been sequenced following CNBr cleavage. Lys-C was unable to cleave the Lys-Trp bond at positions 60-61 and hydrolysis was not particularly efficient at Lys-77 (Fig. 10). This suggests that the tryptophanyl side group may inhibit the binding of Lys-C. The latter protease was highly effective against Lys-15, Lys-37, Lys-56, Lys-69, and Lys-104 (Fig. 10).

All of the Glu/Gln and Asp/Asn residues in the Fraenkel-Conrat et al. (8) sequence have been resolved and several changes have been made in that sequence (Fig. 10). Most significantly, Tyr-61 of the Fraenkel-Conrat sequence could not be found in our protein. Its absence is consistent with the sequences of all known snake venom phospholipases A_2. Position 33 varies with the source of the venom. Venom of C. d. terrificus obtained from the Butantan Institute possessed Gln at position 33, consistent with the Glx reported by Fraenkel-Conrat. However, venom obtained from the Miami Serperntarium yielded Arg at that position with only a trace of Gln. Additional modifications of the Fraenkel-Conrat sequence are as follows: Glx-12=Glu, Asx-16=Asn, Asx-38=Asp, Asx-41=Asp, Asx-48=Asp, Asx-58=Asn, Asx-63=Asp, Glx-83=Glu, Glx-84=Glu, Glx-85=Gln, Glx-88 =Glu, Asx-90=Asp, Glx-95=Glu, Asx-100=Ser, and Asx-106=Tyr. See ref. 9 for experimental details.

(B) ACIDIC SUBUNIT. The acidic subunit of crotoxin consists of three chains, interconnected by disulfide bonds. After reduction and carboxyamidomethylation, the chains were readily separable by reverse-phase HPLC (Fig. 11), and located by a post-column fluorescamine detection system that detects free primary amines. C-chain, which lacks Lys, was undetectable if deblocking of its amino-terminal end with pyroglutamate aminopeptidase was omitted (2). It contains 14 residues (Fig. 12), as anticipated from composition data (19).

Comparisons with 3251 sequences showed strong homologies to portions of snake venom phospholipases A_2 (Fig. 13). Sequence homologies between the acidic subunit and nontoxic crotalid phospholipases yield Z scores of 16.4, 12.0, and 7.5 for the A, B, and C chains, respectively (2).

The A chain has retained both the Tyr-Gly-Cys-Tyr-Cys-Gly-(Trp)-Gly-Gly segment (positions 24-32), common to all known snake venom phospholipases, and the Asp-Arg-Cys-Cys-Phe-(Val)-His-Asp-Cys-Cys-Tyr-(Gly) segment (positions 41-52), which is invariant among crotalid and viperid phospholipases. B chain homologies with phospholipase segments are also very high (positions 71-104, Fig. 13) Locations of four to

five cysteine residues have been maintained, two of which occur in the highly conserved segment Cys-Asp-Lys-Ala-Ala-Ala-Ile-Cys-Phe-Arg (positions 90-99). The 14-residue C chain is homologous with the carboxy terminus of viperid and crotalid phospholipases, retaining both cysteines at positions 118 and 125 (Fig. 13). It also shows high homology with a segment (residues 48-61) of mammalian neurophysins and their precursors. Experimental details relevant to sequencing of the acidic subunit may be found in ref. 2.

V. SPECTRAL MEASUREMENTS. Calculated and experimental molar extinction coefficients were determined for crotoxin, its subunits, and Mojave toxin (Table 10). These values were used for determining concentrations of the various toxins and subunits examined by circular dichroism and fluoresence spectroscopy. Most of these spectral determinations have now been completed and data analysis are in progress. We are currently making modifications on the deconvolution FTIR instrument, which should permit us to make measurements with much smaller protein samples. All spectral measurements will be published together.

VI. CRYSTALS OF MOJAVE TOXIN. Mojave toxin is the major presynaptic neurotoxin present in the venom of the Mojave rattlesnake (C. s. scutulatus). We have shown it to be very similar to crotoxin. Single crystals suitable for x-ray structure analysis have been prepared by Dr. Keith Ward (Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C.) from Mojave toxin purified by a combination of gel filtration and ion exchange chromatography. These crystals were obtained from 10 µl aliquots containing 0.66% acetic acid, 0.34% pyridine, and 53 mg/ml of purified toxin. Aliquots, initially at 55° C, were set up on glass coverslips as hanging droplets, and equilibrated at room temperature over a 1 ml reservoir containing acetic acid and pyridine at concentrations identical to those in the droplet. Best crystals were obtained by seeding these droplets with microcrystals from earlier experiments.

The crystals grow as rectangular plates, occasionally as large as 0.8 x 0.5 x 0.2 mm. They diffract to a resolution better than 2.5 Angstroms, exhibit space group symmetry, $P2_12_12_1$, and have lattice constants a=38.6, b=69.9, c=77.6 Angstroms. There is a single heterodimer in the asymmetric unit. These crystals are thus excellent candidates for a successful, single crystal x-ray diffraction analysis. Data collection is in progress, and it is intended to use the isomorphous replacement technique for analysis as well as molecular replacement methods based on the presumed structural homology between the basic subunit and Crotalid phospholipase A_2.

VII. CROSSLINKING OF THE SUBUNITS OF CROTOXIN. We have nearly completed screening the reaction of four different crosslinkers with crotoxin. These include dimethylsuberimidate (DMS), ethylene glycolbis(succinimidylsuccinate) (EGS), disuccinimidyl tartarate (DST), and the water soluble carbodiimide 1-ethyl-3-(3-dimethyl-aminooropyl) carbodiimide (EDC). We have concentrated on optimizing the extent of crotoxin subunit crosslinking with each of these reagents, and have had reasonable success with all but EGS. We have achieved only low levels of subunit crosslinking with this reagent under a variety of conditions. As such, we will now concentrate our efforts on the other three. Preliminary results indicate that crosslinking of the subunits does reduce crotoxin's toxicity. We need to purify the different crosslinked products and accurately determine LD_{50}'s, along with LD_{50}'s of the appropriate controls (subunits modified by monofunctional reagents that are Lys specific, but that do not physically

crosslink). DST is a cleavable crosslinker, with adjacent hydroxy groups susceptible to periodate (0.015 M) or other oxidizing reagents. This unique crosslinker may provide us with definitive evidence on whether crotoxin dissociation is essential for toxicity.

VIII. CLONING. We have conducted two separate experiments, in collaboration with Dr. Leonard Smith (Pathology Division, USAMRIID), in attempting to clone the crotoxin gene. Both experiments followed similar protocol which is briefly described below. An adult C. vegrandis was milked, four days later sacrificed, and its venom glands dissected and quickly frozen in liquid nitrogen. The glands weighing about 0.5 g were subsequently extracted for total glandular RNA using the phenol procedure and teflon-glass homogenization. Poly A containing RNA was enriched by passage over an oligo (dT) column and used for cDNA cloning. Enriched poly A containing RNA was annealed to a puc9 plasmid vector following lenearization with restriction enzyme Pst I and addition of oligo (dT) tails using terminal transferase. The primary copy from the messenger RNAs was catalyzed by AMV reverse transcriptase. Second strand synthesis was performed using RNAse H to remove the RNA and DNA pol I to copy the first strand. T_4 DNA ligase joined the ends and the constructed molecules were used to transform E. coli. A number of clones were randomly picked, grown up, plasmid DNA extracted, and restricted with a double digest of Hind III and Bam HI to check the size of the inserts. When run on gels, we observed that the inserts were very large and had similar size. We synthesized three oligonucleotide probes to three different areas of the toxin; one to the acidic subunit and two to the basic subunit. These were labeled with ^{32}P and used to probe some of the clones. Six clones grown up on nitrocellulose filters were positive. Growth characteristics and ELISAs run on these different clones suggested that the crotoxin gene had been cloned and that it was being expressed, but not processed by E. coli. Subsequent subcloning and sequencing of pertinent regions of the cloned snake DNA did not reveal the presence of the crotoxin gene.

We need to repeat the cloning attempts. Our most likely problem is low efficiency of mRNA extraction from the venom gland. Various methods for improvement of this process are now being systematically examined in Dr. Smith's laboratory.

DISCUSSION

Double immunodiffusion and ELISA studies demonstrated great antigenic similarity between major neurotoxins isolated from C. d. terrificus, C. s. scutulatus, and C. v. concolor venoms. Intact toxins and basic subunits of heterodimeric proteins showed clear immunochemical identity on double immunodiffusion plates. Intact toxins also contained determinants that were recognized by antisera to their subunits.

It was necessary to add detergent to gels in order to observe precipitin bands in agar when using any of the basic subunits. In its absence, the 'sticky' basic subunit was unable to diffuse out of the well. This probably accounts for the lack of precipitin lines in reports of earlier investigators with the basic subunit (17). No precipitin lines were observed between isolated acidic subunits and any antiserum. This may be due to small size and limited epitopes on the acidic subunit, which prevents precipitable complex formation. Acidic subunit appears less immunogenic than either the basic subunit or intact toxin, since acidic subunit antisera raised against homologous antigen had much lower titers in the ELISA compared with other antisera.

Based on our recent sequence data for the acidic subunit of crotoxin (2) and its similarity to the basic subunit, as well as phospholipase A_2 from C. adamanteus, we were interested in looking at the cross-reactivity of these purified proteins with antisera raised to each of the subunits of crotoxin. When reacted against their homologous antigens, basic subunit antisera

reacted strongly and acidic subunit antisera reacted only moderately. When basic subunit antisera was cross-reacted with acidic subunit and acidic subunit antisera was cross-reacted with basic subunit, both showed moderate levels of cross-reactivity (Table 1). The reduced heterologous reaction seen between basic subunit antisera and acidic subunit is not too surprising, since the acidic subunit lacks three peptides present in the basic subunit protein (2). We also observed cross-reaction between both subunit antisera and phospholipase A_2 from both C. adamanteus and C. atrox venoms. Phospholipase A_2 from these two species differ by only six amino acids. Interestingly, both of these non-toxic, homodimeric phospholipases A_2 showed greater reactivity in ELISA to intact crotoxin antisera than to crotoxin subunit antisera (Table 1). This may indicate that, during complex formation, cross-reactive antigenic sites are created in intact crotoxin. These sites would therefore be similar to the non-toxic, homodimeric phospholipases A_2. This in turn suggests similar quaternary structures for non-toxic, homodimeric phospholipases A_2 and intact, heterodimeric crotoxin. Higher-ordered structures for the acidic subunit of crotoxin appear able to accommodate loss of the three peptide regions without a significant reorganization of tertiary structure proposed to be present in the basic subunit. Neurophysin, which shows homology with the C-chain of the acidic subunit (2), did not react with our antisera, nor did purified phospholipases A_2 from A. mellifera and N. n. atra venoms. No precipitin bands were seen in agar with the phospholipases A_2 from C. adamanteus and C. atrox venoms.

Henderson and Bieber (20) saw no ELISA cross-reaction between rabbit polyclonal antisera raised against the basic subunit of Mojave toxin and venoms of either C. adamanteus or C. atrox. Their inability to detect a cross-reacting component in these crude venoms may simply be a concentration effect. We would expect their antisera to cross-react with a purified sample of phospholipase A_2 from these species.

Earlier work showed that one ml of antisera against reconstituted crotoxin, preincubated with either crotoxin or Mojave toxin, would effectively neutralize both toxins (160 LD_{50} and 80 LD_{50}, respectively) in mice (17). Also, antiserum against the basic subunit of crotoxin was shown to be effective in neutralizing intact crotoxin (i.p., 45 LD_{50}) (18). We have confirmed and extended these observations by showing in one series of experiments that antisera against intact crotoxin and the basic subunit neutralized 330 and 460 LD_{50} of crotoxin per ml of serum, respectively. Both were an order of magnitude more effective, than antiserum raised against the acidic subunit. Antisera against intact crotoxin neutralized nearly equivalent total toxin doses of concolor toxin and Mojave toxin (Fig. 3).

While antisera against intact crotoxin and basic subunit of crotoxin had high neutralization capacities against crotoxin when mixed before injection, such was not the case when i.v. injection of toxin was followed by i.v. neutralizing antiserum. We found that, unless neutralizing antisera were injected within the first min after crotoxin injection, survival was rare (Table 2). This indicates that crotoxin is rapidly removed from the circulation and becomes unavailable to neutralizing antibodies. Loss from the circulatory system must be rather selective however, since the amounts injected (2 LD_{50}) are too small to permit random losses and still promote the high mortality that we observe. This may be an indication that, once the toxin binds to the presynaptic terminal site, it becomes unavailable for neutralization by antiserum--perhaps as a result of toxin internalization. When crotoxin was injected i.m. (a condition more representative of snake bite) neutralizing antiserum was considerably more

effective in preventing death, with most animals surviving that received antiserum within 30 min (Table 3).

Four different monoclonal antibodies have been purified from mouse ascites fluid by a combination of ammonium sulfate precipitation and protein A-Sepharose column chromatography. We are presently characterizing these antibodies to determine whether they recognize the same antigenic site or have partial overlap, in addition to determining their cross-reactivity with different related toxins and venoms. One of the four has proven to be an excellent neutralizer of crotoxin, with about 1.6 mols of crotoxin neutralized per mol of antibody. This has led to a more extensive examination of its neutralizing ability of both purified crotoxin and different crude venoms.

On a per weight or molar basis, it is over 50-times more effective than commerical Wyeth polyvalent crotalid antivenin in neutralizing crotoxin. When added to Wyeth antivenin, it enhances its neutralizing ability toward crude venom from C. d. terrificus four-fold. It was not effective in neutralizing crude C. d. terrificus lethality. In fact, it appeared to potentiate crude venom toxicity at crude venom doses slightly above the crude venom LD_{50} in certain animals and promoted a bizarre rolling action in the animals in addition to hyperactivity. No such effects were noted when crude Mojave venom was neutralized with the monoclonal antibody, suggesting that the South American rattlesnake venom contains a second toxic component that is absent (or present in much lower concentrations) in Mojave rattlesnake venom. Purified concolor toxin is neutralized with the monoclonal antibody, but less effectively than crotoxin. Preliminary competitive studies using biotinylated crotoxin also indicate that concolor toxin reacts with the neutralizing monoclonal antibody somewhat less efficiently than it does with crotoxin.

The gel filtration profile reported here for C. vegrandis venom on Sephacryl S-200 (Figure 6) is very similar to that obtained by Scannone et al. (21) using Sephadex G-100. In both cases Peak 3 appeared equivalent. The former authors reported that Peak 3 was the only fraction recovered that had a more toxic i.v. LD_{50} in mice than crude venom. Based on its LD_{50} and position of elution, it was suggested that C. vegrandis venom contained crotoxin. In our hands the principal toxic component also eluted from the gel filtration column in the same manner as crotoxin.

During FPLC, vegrandis toxin exhibited several isoforms, which is characteristic of crotoxin and Mojave toxin (5,22,23). Further, vegrandis toxin spontaneously dissociated into subunits, as noted earlier with crotoxin from certain lots of C. d. terrificus venom. We confirmed that the ultraviolet absorbing material eluting in the void volume from the Mono Q column (Figure 9) was the basic subunit of vegrandis toxin by its cross-reaction with crotoxin antiserum.

Double immunodiffusion assays with the three separable isoforms of vegrandis toxin from Mono Q (Figure 7) demonstrated immunological identity between the isotoxins and with crotoxin. In addition, toxicity determinations confirm that two of the three peaks had LD_{50}-values similar to that of crotoxin. SDS PAGE behavior of vegrandis toxin is likewise consistent with other crotalid heterodimeric presynaptic neurotoxins (5). The weaker reactivity of Peak 1, combined with its reduced toxicty suggests the presence of a contaminating protein. This may also be indicated by SDS PAGE in which the "basic subunit" band was less discrete than in other samples. One possible explanation is that Peak 1 is contaminated by a non-toxic homodimeric phospholipase A_2, such as that from C. atrox or C. adamanteus. The non-toxic phospholipases elute closely to the presynaptic neurotoxins on Mono Q, but non-toxic phospholipases do not dissociate spontaneously as do the heterodimeric phospholipases. Furthermore, when examined on SDS PAGE, non-toxic phospholipases appear as a single band

with a mobility that is very close to that of the basic subunit from crotalid presynaptic neurotoxins (AIRD and KAISER, unpublished data).

Venom of C. v. lutosus was fractionated over the gel filtration medium Sephacryl S-200. Venom proteins were resolved into five major fractions. Phosphodiesterase and L-amino acid dehydrogenase eluted in the first fraction with estimated molecular wieghts of 159kD and 133 kD, respectively. Phospholipase activity eluted as a major peak in the second and third fractions with a trailing peak of activity between the third and fourth fractions, with estimated molecular weights of 50.4 kD and 22.2 kD, respectively. These estimates approximate those anticipated for homotetramers and homodimers, and constitute the first suggestion that crotalid phospholipases may achieve greater structural complexity in solution than the dimeric state. Protease activity, as evidenced by hydrolysis of six natural and synthetic substrates, was distributed throughout the first four fractions, suggesting the presence of multiple proteases with a wide range of molecular weights and with differing substrate specificities. Small myotoxins are absent from the venom of these populations. This is consistent with data from two Utah C. v. lutosus populations, but contrasts with all existing information on the venoms of conspecific taxa C. v. viridis, C. v. concolor, and C. v. helleri. Based on experience with other rattlesnake venoms, the fifth S-200 fraction probably consists of hypotensive peptides. No evidence was found for the presence of a crotoxin homolog in this venom.

An amino acid sequence comparison was carried out with the basic subunit of crotoxin and with 3447 sequences in the Protein Identification Resource at NBRF, which yielded a mean similarity score of 21.2, and 532 sequences with a score greater than 27. The five most similar sequences were phospholipases A_2 from crotalid and viperid venoms, with similarity scores ranging from 436 down to 388. Nineteen of the next 22 most closely related sequences were phospholipases A_2 from elapid venoms (similarity scores from 267 to 192).

The basic subunit of the crotoxin homolog from the venom of C. v. concolor displays 100% homology with the basic subunit of crotoxin over the N-terminal 43 residues. Percentage homologies with the acidic subunit of crotoxin and with other crotalid and viperid phospholipases are as follows: acidic subunit, 56.6% (estimated over 76 known residues of the acidic subunit); Bitis caudalis phospholipase, 52.5%; Crotalus adamanteus phospholipase, 50.0%; Crotalus atrox, 49.2%; Bitis nasicornis phospholipase, 49.2%; Bitis gabonica phospholipase, 47.5% and Trimeresurus okinavensis phospholipase, 47.2%. There is greater similarity between the acidic subunit of crotoxin and nontoxic, homodimeric phospholipases from C. adamanteus and C. atrox than between the two crotoxin subunits. The basic subunit of crotoxin and its homolog from the venom of C. v. concolor show greater similarity with the toxic monomeric phospholipase A_2 from B. caudalis than with any known crotalid phospholipases. This implies that toxicity evolved in phospholipases prior to the divergence of the Crotalidae and the Viperidae. Furthermore, the development of toxicity may have preceded the development of homo- and/or heterodimerism in phospholipases.

Attempts to complete the B-chain sequence of the acidic subunit of crotoxin are presently underway; however, this cannot be accomplished by common methods, such as a combination of aminopeptidase and carboxypeptidase. This is because the unsequenced portion is ten residues long, is blocked at the amino terminus by an unknown agent, and contains five Glx and three Asx. Of the ten residues not sequenced, five can be positioned with considerable certainty on the basis of probable ancestral sequences. These include Gln-71, Asx-72, Gly-73, Val-76, and Cys-77.

Sequence positions 24-32, Fig. 13, near the amino terminus of the A-chain is at the interface between monomers in homodimeric phospholipases A_2 and may constitute part of the subunit binding site. Sequence positions 41-52, Fig. 13, contain the phospholipase A_2 active

site and Asp-48, which is specifically implicated in Ca^{2+} binding. Homology of the C-chain is maximal with phospholipase A_2 from the venom of the eastern diamondback rattlesnake (C. adamanteus)(Z=7.5).

All cysteine residues common to both toxic and nontoxic phospholipases appear to have been retained in the acidic subunit. It seems probable, therefore, that the disulfide bonds correspond with assignments made by Heinrikson for group II phospholipases (24), as shown in Fig. 12.

The low pI of the acidic subunit (3.2 as opposed to 9.7 for the basic subunit), has been produced partly by the exclusion of three segments (positions 1-22, 62-70, and 105-111; Fig. 13) containing a total of seven basic residues and only one to two acidic residues. These segments are believed to interact with phospholipid substrates in intact, nontoxic, crotalid phospholipases (J. Maraganore, personal communication). From the sequence comparison data it is not possible to determine the amino acid residues involved in the cleavages to generate the three chains. Clearly, it will be important to determine the sequence of the whole precursor in order to assign the sites of cleavage. It will be of interest to know how these cleavages relate to cleavages of precursors of other bioactive peptides.

Homology of both the acidic and basic subunits is greatest with nontoxic, crotalid phospholipases, which exist in solution as stable dimers with dissociation constants of 10^{-9}-10^{-11} M . Crotoxin exhibits maximal toxicity only as a dimer, despite the fact that it dissociates upon reaching the target tissue. That is, neurotoxicity is reduced about 10-fold if the dissociated basic subunit is injected alone. While retention of the subunit binding site by the acidic subunit further suggests its origin from a homodimeric phospholipase and suggests that the two active sites of the subunits face on another as proposed (25), the loss of enzymatic activity in the acidic subunit is intriguing. Randolph et al. (26) report that the dimeric phospholipase A_2 from C. atrox is completely destabilized when the amino-terminal 10 residues of the protein are removed with cyanogen bromide. In the acidic subunit, 22 residues are missing from the amino terminus.

Studies on crotoxin and related homologs in the areas of crystal structure, cross-linking, and cloning are in preliminary stages. Brief discussions of early findings were presented in each of these areas in the RESULTS section.

CONCLUSIONS

(1) Antisera were raised against intact crotoxin (Crotalus durissus terrificus), Mojave toxin (Crotalus scutulatus scutulatus), and concolor toxin (Crotalus viridis concolor), as well as the subunits of crotoxin. Double immunodiffusion and enzyme-linked immunosorbent assays (ELISA) demonstrated antigenic similarity between these three purified toxins and their subunits. Additionally, when crotoxin antisera were preincubated with each of the three toxins before injection, the lethal activity of all were neutralized equally well. Antiserum was considerably more effective in neutralizing crotoxin in vivo when the toxin was injected intramuscularly, than when injected intravenously. Antisera against both intact crotoxin and its basic subunit were an order of magnitude more effective than crotoxin acidic-subunit antiserum in crotoxin neutralization. Purified phospholipase A_2 from Crotalus adamanteus and Crotalus atrox showed weak cross-reactivity with antisera raised against intact crotoxin and its subunits in the ELISA. Our results suggest that crotalid neurotoxins can be detected and neutralized by polyclonal antibodies raised against any intact toxin or basic subunit in this group of homologous toxins.

(2) We have generated, purified and partially characterized four different monoclonal antibodies against crotoxin. One of these is a potent neutralizing antibody against this toxin.

(3) Crotalus vegrandis venom was shown to contain a toxic protein that is structurally and antigenically similar to the major neurotoxins from C. d. terrificus, C. s. scutulatus, and C. v. concolor. It also appears likely that this toxin is the product of a duplicated locus as has been suggested for crotoxin (23).

(4) Great Basin rattlesnake (C. v. lutosus) venom has been initially fractionated and characterized. While it contains many enzymes characteristic of crotalid venoms, we found no evidence for the presence of a presynaptic neurotoxin.

(5) The complete amino acid sequence of the basic subunit of crotoxin from the venom of C. d. terrificus has been determined. Sequence comparisons between the basic and acidic subunits of crotoxin and between the basic subunit and other phospholipase A_2 molecules indicate that the basic subunit is structurally most similar to the monomers of nontoxic, dimeric phospholipases A_2 from the venoms of C. adamanteus, C. atrox, and T. okinavensis, and to the toxic monomeric phospholipase A_2 from the venom of B. caudalis.

(6) The complete sequences of two of the three acidic subunit chains of crotoxin, from the venom of C. d. terrificus have been determined. In addition, all but the ten amino-terminal residues of the third chain have been sequenced. Sequence comparison data suggest that the acidic subunit has been derived from a nontoxic, homodimeric, crotalid phospholipase A_2. When compared with sequences of phospholipases A_2, the acidic subunit lacks a 22-residue amino-terminal segment and two additional segments that are implicated in phospholipid substrate binding. However, it apparently retains an intact active site, the calcium binding loop, and segments involved in subunit binding in homodimeric phospholipases A_2. Crystals suitable for X-ray diffraction studies with both crotoxin and Mojave toxin have recently been produced; thus with these sequencing data it should now be possible to determine the three-dimensional structure of the intact neurotoxin and dissociated subunits.

(7) Preliminary results on the crystallization of Mojave toxin and the cross-linking of crotoxin are quite promising. We expect useful information on the higher-ordered structure of Mojave toxin to result from our collaborative studies with Dr. Keith Ward as well as our spectral studies. Initial cross-linking conditions with crotoxin have been optimized with three different cross-linkers. We are now progressing to the examination of the properties of these cross-linked products.

(8) Attempts to clone the crotoxin gene in two separate experiments have been unsuccessful. Once we improve our extraction methodology of mRNA from venom glands we will repeat the cloning.

RECOMMENDATIONS

(1) Continue our characterization of the neutralizing monoclonal antibody. This line will provide us with material to attempt to prepare anti-idiotype monoclonal antibodies for possible use as an anti-idiotype vaccine in collaboration with Dr. John L. Middlebrook. If we can identify the neutralizing monoclonal antibody epitope on crotoxin, we may be able to prepare a synthetic peptide that would reflect its structure and also serve as a non-toxic immunogen for crotoxin and related presynaptic neurotoxins.

(2) Continue our spectral studies on crotoxin, its subunits, and related homologs, that are currently in progress.

(3) Continue our collaboration with Dr. Keith Ward on the determination of the crystal structure of Mojave toxin that is also in progress.

(4) Continue the cross-linking studies on crotoxin that are underway, in an effort to determine whether crotoxin subunit dissociation is essential for neurotoxicity.

(5) Continue to attempt to sequence the ten-amino acid amino-terminal fragment of the B-chain of crotoxin's acidic subunit.

(6) Attempt to establish an in vitro system for examining the biological effects of presynaptic neurotoxins.

(7) Once we develop improved methodology for mRNA extraction from venom glands, repeat the attempt to clone the crotoxin gene.

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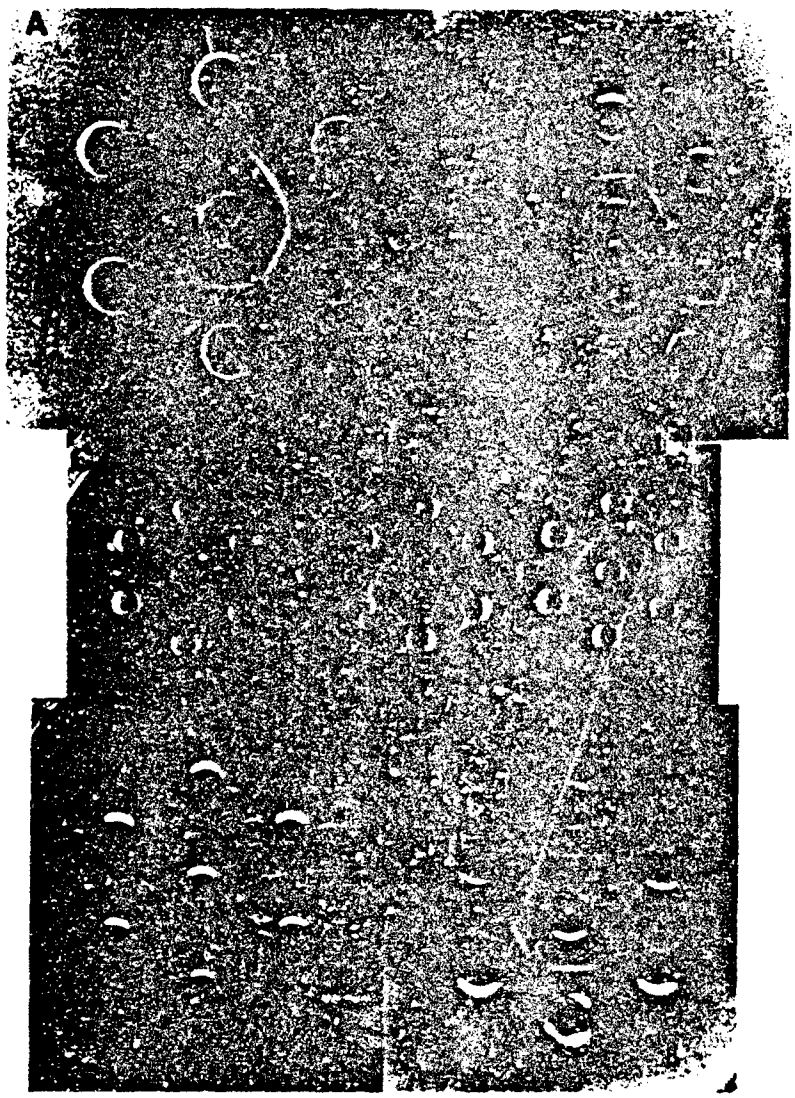


FIG. 1. OUCHTERLONY AGAR GEL-DIFFUSION PLATES.
(A) Center well: anti-crotoxin serum. Peripheral wells contained crotoxin at the following concentrations (mg/ml): (1) 0.5; (2) 0.25; (3) 0.12; (4) 0.06; (5) 0.032; (6) 0.016. (B) Center well: anti-crotoxin serum. Peripheral wells: (1, 2) crotoxin; (3, 4) *concolor* toxin; (5, 6) Mojave toxin. All toxins were at a concentration of 0.125 mg/ml. (C) Center well: crotoxin basic subunit (0.125 mg/ml). Peripheral wells: (1, 2) anti-crotoxin serum; (3, 4) anti-*concolor* toxin serum; (5, 6) anti-Mojave toxin serum. (D) Center well: *concolor* toxin basic subunit (0.125 mg/ml). Peripheral wells were as (C). (E) Center well: Mojave toxin basic subunit (0.125 mg/ml). Peripheral wells were as (C). (F) Center well: crotoxin basic subunit antiserum. Peripheral wells: (1, 2) basic subunits of crotoxin; (3, 4) *concolor* toxin; (5, 6) Mojave toxin. All toxins were at a concentration of 0.125 mg/ml. (G) Center well: intact crotoxin (0.125 mg/ml). Peripheral wells: (1, 2) crotoxin acidic subunit antiserum; (3, 4) crotoxin basic subunit antiserum; (5, 6) intact crotoxin antiserum.

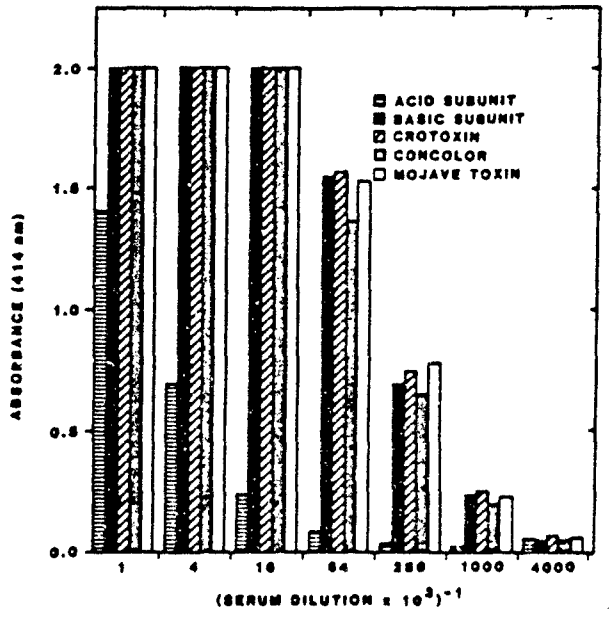


FIG. 2. ELISA TITRATION OF CROTOXIN ANTISERUM AGAINST CROTOXIN, *concolor* TOXIN, MOJAVE TOXIN AND THE ACIDIC AND BASIC SUBUNITS OF CROTOXIN.
Titrations were performed as described in Materials and Methods. A serum dilution of 1 on the abscissa corresponds to a dilution of 1/1000, 4 corresponds to 1/4000, etc.

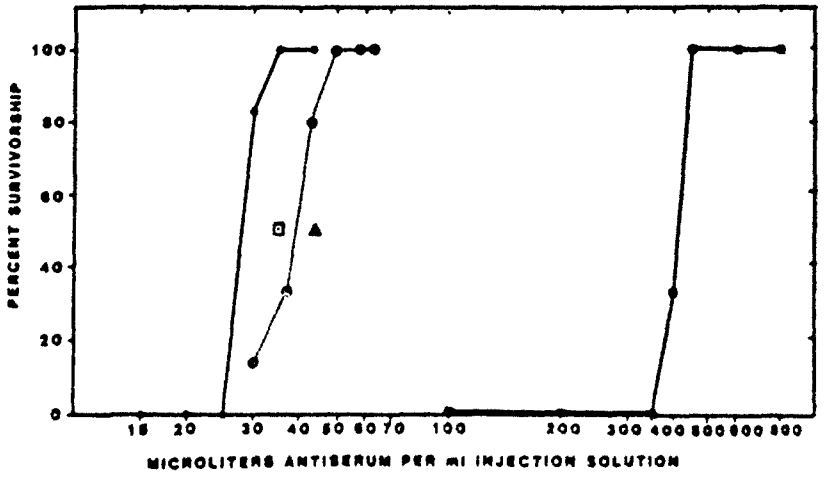


FIG. 3. NEUTRALIZATION OF INTACT CROTOXIN BY ANTISERUM RAISED AGAINST CROTOXIN AND EACH OF ITS SUBUNITS.
—⊙— Intact crotoxin antiserum; —●— crotoxin basic subunit antiserum; —■— crotoxin acidic subunit antiserum. LD_{50} (i.v.) of crotoxin was 0.056 μg/g in male mice. When an equivalent LD_{50} dose of Mojave toxin (⊡) or *concolor* toxin (▲) was used, the indicated neutralization midpoints using crotoxin antisera were obtained.

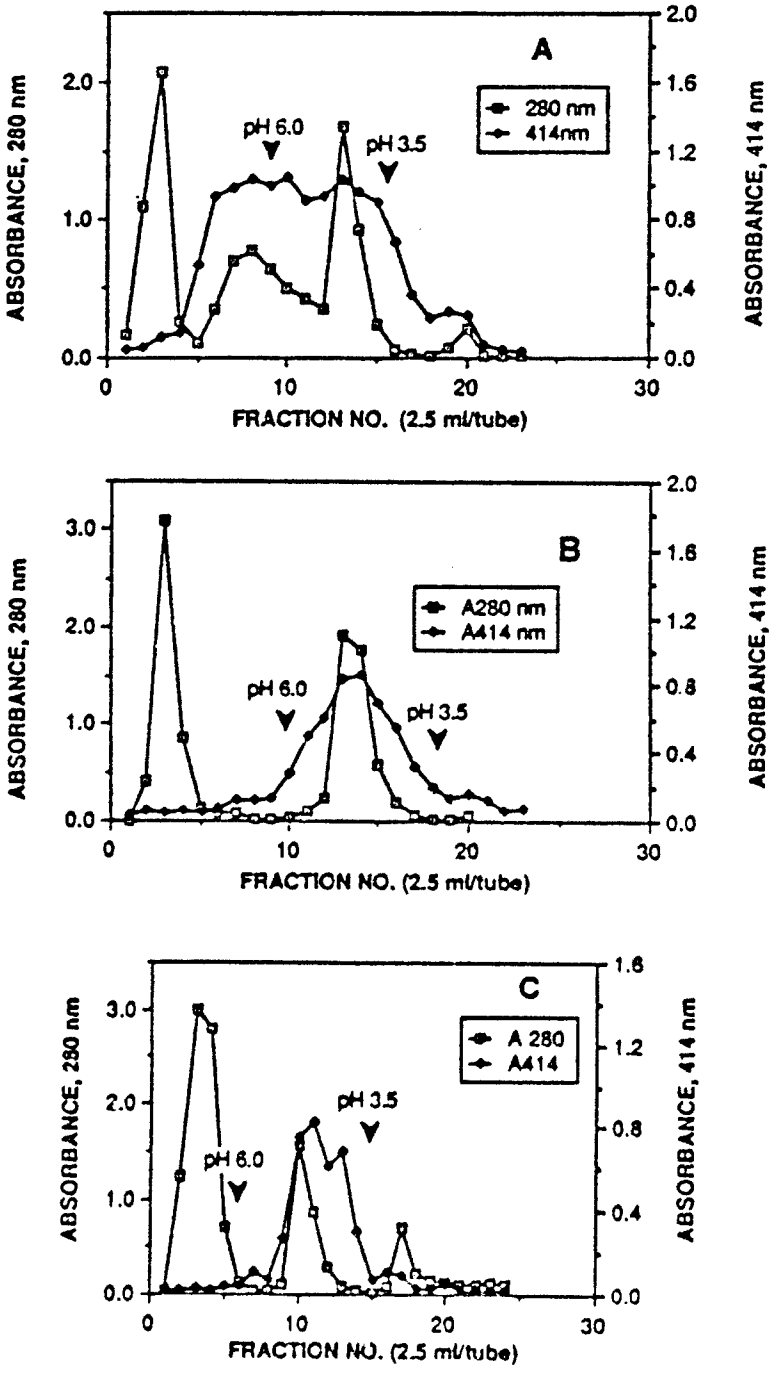


Fig. 4. PROTEIN A-SEPHAROSE CHROMATOGRAPHY OF MONOCLONAL ANTIBODIES 1, 5, AND 11 CORRESPOND TO PANELS A, B, AND C, RESPECTIVELY. Dialyzed, ammonium sulfate precipitate from two ml of ascites fluid prepared from mice innoculated with the respective hybridomas were chromatographed on 1 X 7 cm columns as described in Materials and Methods. Protein elution was monitored at 280 nm. Aliquots from each fraction were removed, ELISAs run on crotoxin-coated microtiter plates, and absorbancies determined at 414 nm on a Titertek Multiskan MC.

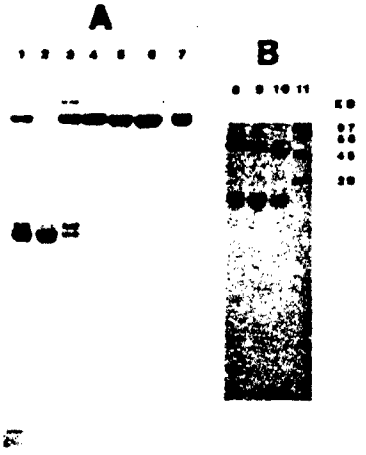
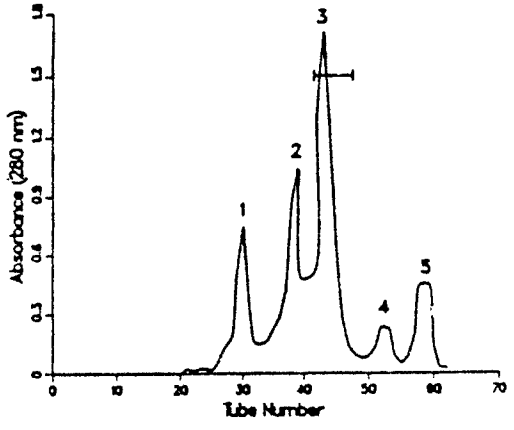


Fig. 5. ELECTROPHORETIC ANALYSIS OF MONOCLONAL ANTIBODIES. (A) Polyacrylamide gel (7.5%) in the absence of reducing agent and stained with Coomassie blue. (B) Polyacrylamide gel (15%) of protein A purified monoclonal antibodies reduced in dithiothreitol immediately before electrophoresis and stained with Coomassie blue.
Well 1: Crude ascites fluid containing monoclonal antibody sample 1.
Well 2: Ammonium sulfate supernatant of ascites fluid containing monoclonal antibody sample 1.
Well 3: Ammonium sulfate precipitate of ascites fluid containing monoclonal antibody sample 1.
Wells 4, 5, 6, and 7: Monoclonal antibody samples 1, 2, 5, and 11, respectively, after chromatography on protein A-Sepharose. Wells 8, 9, and 10: Monoclonal antibody samples 1, 2, and 5. Molecular weight values for markers run in well 11 are noted.

Fig. 6. GEL FILTRATION OF CRUDE VENOM FROM A POOLED SAMPLE OF CROTALUS VEGRANDIS ON SEPHACRYL S-200. Crude venom (0.6 ml) was passed over a 2.5 x 94 cm column equilibrated with 0.1M sodium acetate (pH 4.0). Protein concentrations were determined by absorbance at 280 nm. Tubes 42-48, which constituted ~41% of the material applied, were pooled for subsequent chromatography on Mono Q.



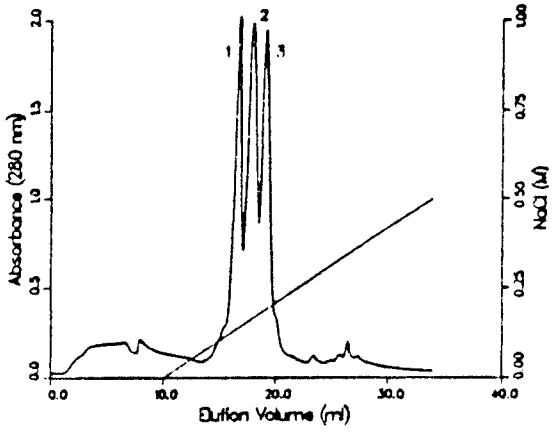


Fig. 7. ANION EXCHANGE COLUMN CHROMATOGRAPHY OF POOLED FRACTIONS FROM THE SEPHACRYL S-200 RUN. Peak 3 from Fig. 1 was pooled, dialyzed against deionized water, and lyophilized. About 8 mg was redissolved in 5 ml of 50mM Tris-HCl (pH 7.2) and applied to a Mono Q column and eluted with a linear gradient of NaCl in the above buffer. The three main peaks were pooled as indicated, dialyzed against deionized water, and lyophilized.

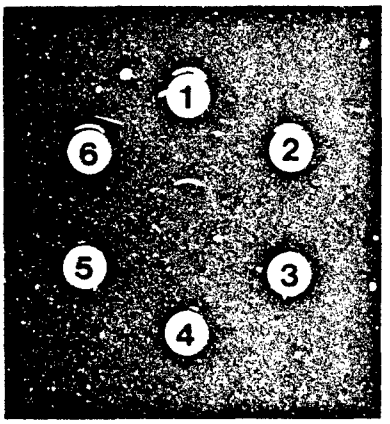


Fig. 8. OUCHTERLONY AGAR GEL-DIFFUSION PLATE. Center well contains intact crotoxin rabbit antiserum. Peripheral wells: (1) buffer only; (2) intact crotoxin; (3) peak 1, Fig. 2; (4) peak 2, Fig. 2; (5) peak 3, Fig. 2; (6) intact crotoxin. All samples were at a concentration of 0.125 mg/ml.

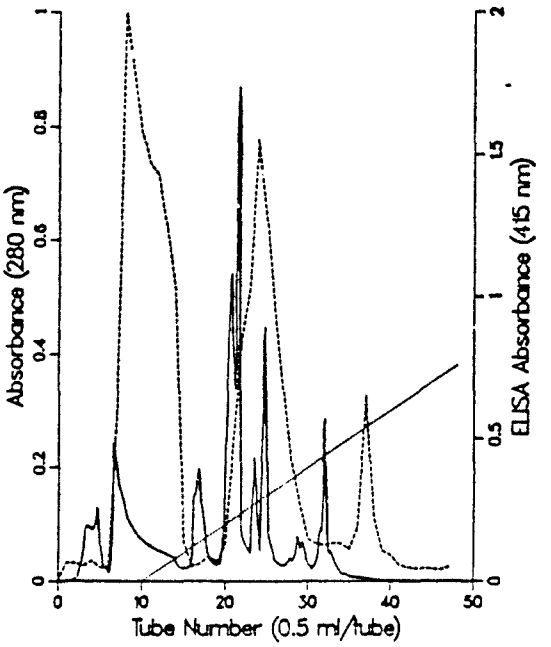


Fig. 9. ABSORBANCE ELUTION PROFILE (280 nm) OF VENOM FROM AN INDIVIDUAL JUVENILE CROTALUS VEGRANDIS (SOLID LINE) COUPLED WITH A DOUBLE ANTIBODY SANDWICH ELISA RESPONSIVE TO CROTOXIN-RELATED PROTEINS (DASHED LINE - 415 nm). About25 µl of crude venom was dissolved in 1.0 ml of 20mM Tris-HCl (pH 8.4), filtered, and chromatographed on a Mono Q column. Aliquots (5µl) from each fraction was assayed for immunological identity with crotoxin.

```
          1         5         10        15        20        25        30
F-C       H-L-L-Q-F-N-K-M-I-K-F-Z-T-R-K-B-A-I-F-F-Y-A-F-Y-G-C-Y-C-G-W-
N-terminal H-L-L-Q-F-N-K-M-I-K-F-E-T-R-K-N-A-I-P-F-Y-A-F-Y-G-C-Y-C-G-W-
CNBr                        I-K-F-E-T-R-K-N-A-I-P-F-Y-A-F-Y-G-C-Y-C-G-W-

                  35        40        45        50        55        60
F-C       G-G-Z-G-R-P-K-B-A-T-B-R-C-C-F-V-H-B-C-C-Y-G-K-L-A-K-C-B-T-K-
N-terminal G-G-*-G-R-P-K-D-A-T-D
CNBr      G-G-*-G-R-P-K-D-A-T-D-R-C-C-F-V-H-D-C-C
Arg-C                   P-K-D-A-T-D-R-C-C-F-V-H-D-C-C-Y-G-K-L-A-K-C-N-T-K-
Arg-C                     (R)D-A-T-D-R-C-C-F-V-H-D-C-C-Y-G-K-L-A-K-C-N-T-K-
Lys-C                                                       C-N-T-K-

                        65        70        75        80        85
F-C  (Y-) W-B-I-Y-R-Y-S-L-*-S-G-Y-I-T-C-G-K-G-T-W-C-Z-Z-Z-I-
Arg-C     W-D-I-Y-R Y-S-L-*-S-G-Y-I-T-C-G-K-G-T-W-C-E-E-Q-I-
Arg-C     W-D-I-Y-R Y-S-L-K-S-G-Y-I-T-C-G-K-G-T-W-C-E-E-Q-I-
Lys-C     W-D-I-Y-R-Y-S-L-R-S-G-Y-I-T-C-G-K G-T-W-C-E-E-Q-I-

                  90        95        100       105       110       115
F-C       C-Z-C-B-R-V-A-A-Z-C-L-R-R-B-L-S-T-Y-K-B-G-Y-M-F-Y-P-D-S-R-C-
Arg-C     C-E-C-D-R V-A-A-E-C-L-R    S-L-S-T-Y-K-Y-G-Y-M-F-Y-P-D-S-R
Arg-C     C-E-C-D-R V-A-A-E-C-L-R
Lys-C     C-E-C-D-R-V-A-A-E-C-L-R-R-S
CNBr                                                  F-Y-P-D-S-R-C-

                  120
F-C       R-G-P-S-E-T-C
CNBr      R-G-P-S-E-T-C
```

Fig. 10. Alignment of cleavage fragments in the sequencing of the basic subunit of crotoxin from the venom of C. d. terrificus. A blank (Arg-98) or the absence of a hyphen (Arg-90, Val-91) indicates the carboxyl end of one cleavage fragment and the amino-terminal end of the next fragment. An asterick (position 33) denotes Gln in some samples and Arg in others. No single lot of venom displayed both forms. The Fraenkel-Conrat sequence is designated F-C. Changes in that sequence are given in boldface letters.

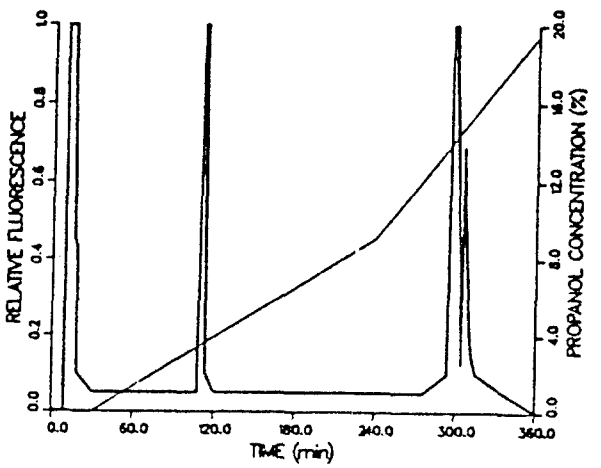


Fig. 11. Elution of the three acidic subunit chains from a C_{18} reverse-phase column. The equilibration buffer was 0.5 M pyridine–acetate, pH 4.0. The elution buffer differed in that it contained 40% 1-propanol. HPLC was carried out at 22 °C with a flow rate of 0.75 mL/min.

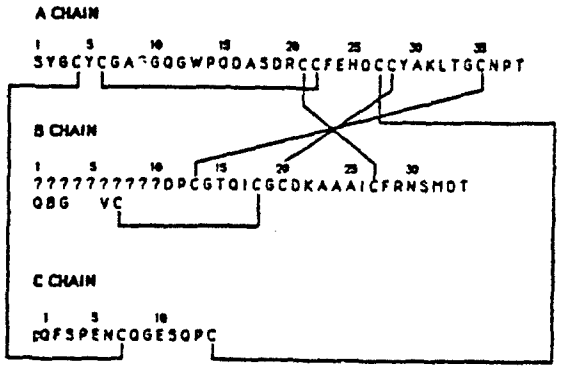


FIGURE12: Primary structures of the acidic subunit chains of the presynaptic neurotoxin from the South American rattlesnake (*C. durissus terrificus*). The B chain is blocked at the amino terminus but probably not with a formyl group or with pyroglutamate. Probable residues in the unsequenced region are shown below the B chain. Cleavage of the B chain was effected with 100% TFA at 60 °C for 2 h. The C chain was blocked with pyroglutamate.

PROTEIN	FAMILY	SPECIES	RESIDUES 1 5 10
Crotoxin Acidic	Crotalidae	*Crotalus d. terrificus*	
Crotoxin Basic	Crotalidae	*Crotalus d. terrificus*	H L L Q F N K M I K
Phospholipase A_2	Crotalidae	*Crotalus adamanteus*	S L Y Q F E T L I M
Phospholipase A_2	Crotalidae	*Crotalus atrox*	S L Y Q F E T L I M
Phospholipase A_2	Crotalidae	*Trimeresurus okinavensis*	H L M Q F E T L I N
Phospholipase A_2	Viperidae	*Bitis caudalis*	N L I Q F G N M I S
Phospholipase A_2	Viperidae	*Bitis nasicornis*	D L T Q F G N M I N
Phospholipase A_2	Viperidae	*Bitis gabonica*	D L T Q F G N M I N

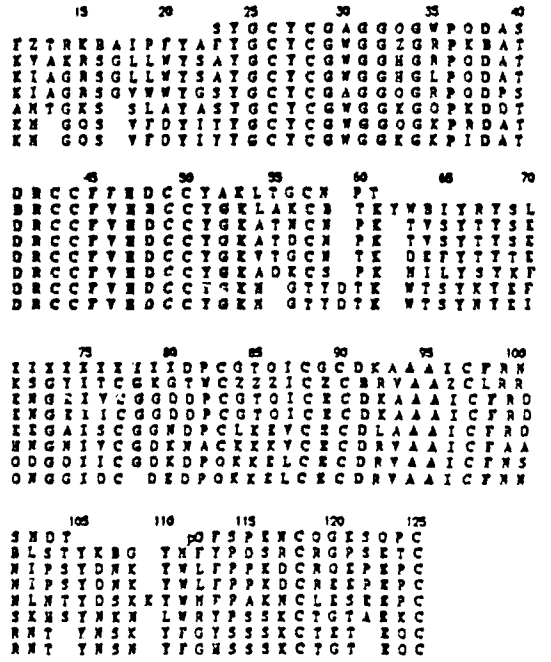


FIGURE13: Sequences of the acidic and basic subunits of crotoxin relative to other crotalid and viperid phospholipase A_2 sequences. The three phospholipase segments consisting of residues 23–61, 71–104, and 112–125 correspond to the acidic subunit A, B, and C chains, respectively.

TABLE 1. REACTIONS* OF VARIOUS PROTEINS TO ANTISERUM RAISED AGAINST THE ACIDIC AND BASIC SUBUNITS OF CROTOXIN, CROTOXIN, MOJAVE TOXIN AND *concolor* TOXIN USING THE ENZYME-LINKED IMMUNOSORBENT ASSAY.

Antigen	Antisera against				
	Acidic subunit	Basic subunit	Intact crotoxin	Intact *concolor*	Intact Mojave toxin
Acidic subunit	+ +	+ +	+ +	+ +	+ +
Basic subunit	+ +	+ + + +	+ + + +	+ + + +	+ + + +
Intact crotoxin	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Intact *concolor*	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Intact Mojave toxin	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
C. d. terrificus venom	+ + +	+ + + +	+ + + +	ND	ND
C. s. scutulatus venom	+ + +	+ + + +	+ + + +	ND	ND
PLA, *C. atrox*	+	+	+ +	ND	ND
PLA, *C. adamanteus*	+	+	+ +	ND	ND
β-Bungarotoxin	trace	trace	trace	ND	ND
Neurophysin	–	–	–	ND	ND
PLA, *Apis mellifera*	–	–	–	ND	ND
PLA, *N. n. atra*	–	–	–	ND	ND

Reactions were scored by using the greatest dilution that provided a reading ≥2.1 times background. Absorbance was read at 414 nm after 10 min reaction at room temperature. Antigen concentration was 1 μg/ml.
*<1/500 –; 1/500 trace; 1/12,500 +; 1/64,000 + +; 1/256,000 + + +; 1/1,000,000 + + + +; ND = not determined.

TABLE 2. *IN VIVO* NEUTRALIZATION BY ANTISERUM OF CROTOXIN INJECTED INTRAVENOUSLY.

Injections	Mortality (deaths/animal number)	
	Crotoxin antisera	Basic subunit antisera
Antiserum injected before crotoxin	0/9	0/4
Crotoxin injected before antiserum		
1 min	2/4	0/4
2 min	6/7	4/5
3 min	6/6	
4 min		2/2

Two LD_{50} of crotoxin (0.112 μg/g) were injected into male mice. Diluted antisera equivalent to 10 LD_{50} neutralizing doses were injected i.v. at the indicated times. Mortality was determined after 24 hr.

TABLE 3. *IN VIVO* NEUTRALIZATION BY ANTISERUM OF CROTOXIN INJECTED INTRAMUSCULARLY.

Injections	Mortality (deaths/animal number)
Toxin only	20/20
Antiserum injected before crotoxin	0/8
Crotoxin injected before antiserum	
5 min	0/4
10 min	0/4
15 min	0/4
30 min	1/8
60 min	3/8
90 min	4/8
120 min	4/8
150 min	6/8
180 min	3/4

Four to six i.v. LD_{50} of crotoxin (0.224 – 0.336 μg/g) were injected into male mice. This was followed by injection (i.v.) of a diluted anti-crotoxin sera equivalent to 10 LD_{50} neutralizing doses, at the indicated times. Mortality was determined after 24 hr.

Table 4. Toxicity of crude C. d. terrificus venom in mice in the absence and presence of monoclonal antibody.

Concentration crude venom	Estimated conc. crotoxin	Concentration of monoclonal antibody sample 1	Deaths/Injected*
0.05 µg/g	0.025 µg/g 0.109 nmols/ml	0.217 nmols/ml	0/4
0.10 µg/g	0.050 µg/g 0.217 nmols/ml	0.435 nmols/ml	3/4
0.125 µg/g	0.0625 µg/g 0.272 nmols/ml	0.542 nmols/ml	4/4
0.125 µg/g	0.0625 µg/g 0.272 nmols/ml	0	3/3
0.150 µg/g	0.075 µg/g 0.328 nmols/ml	0.651 nmols/ml	6/8
0.150 µg/g	0.075 µg/g 0.326 nmols/ml	0	2/2

*24 hours. I.v. LD_{50} of crude C. d. terrificus venom is 0.075 µg/g in male mice.

Table 5. Neutralization of crude C. d. terrificus venom with Wyeth polyvalent antivenin.

Concentration crude venom	Estimated conc. crotoxin	Wyeth polyvalent antivenin	Deaths/Injected*
0.05 µg/g	0.025 µg/g	1.76 mg prot/ml	0/4
0.10 µg/g	0.050 µg/g	3.52 mg prot/ml	0/4
0.15 µg/g	0.075 µg/g	5.28 mg prot/ml	0/4
0.20 µg/g	0.10 µg/g	7.04 mg prot/ml	0/4

*24 hours. I. v. LD_{50} of crude C. d. terrificus venom is 0.075 µg/g in male mice.

Table 6. Toxicity of increasing amounts of crude C. d. terrificus venom and constant relative amounts of Wyeth antivenin.

Concentration crude venom	Estimated conc. crotoxin	Wyeth polyvalent antivenin	Deaths/Injected*
0.30 µg/g	0.15 µg/g	3.32 mg protein/ml	0/4
0.40 µg/g	0.20 µg/g	3.32 mg protein/ml	4/4
0.60 µg/g	0.30 µg/g	3.32 mg protein/ml	4/4
0.80 µg/g	0.40 µg/g	3.32 mg protein/ml	4/4

*24 hours. I. v. LD_{50} of crude C. d. terrificus venom is 0.075 µg/g in male mice.

Table 7. Effects of supplementing Wyeth antivenin with monoclonal antibody 1 in neutralizing crude venom from C. d. terrificus.

Concentration crude venom	Wyeth Polyvalent antivenin	Monoclonal conc/ml injection medium	Deaths/Injected*
0.30 µg/g	3.32 mg prot/ml	195 µg (1.30 nmols)	0/4
0.40 µg/g	3.32 mg prot/ml	292 µg (1.95 nmols)	0/4
0.60 µg/g	3.32 mg prot/ml	390 µg (2.60 nmols)	0/4
0.80 µg/g	3.32 mg prot/ml	585 µg (3.90 nmols)	0/4
1.00 µg/g	3.32 mg prot/ml	650 µg (4.33 nmols)	0/5
1.20 µg/g	3.32 mg prot/ml	780 µg (5.20 nmols)	1/5
1.40 µg/g	3.32 mg prot/ml	909 µg (6.06 nmols)	4/4

*24 hours. I. v. LD_{50} of crude C. d. terrificus venom is 0.075 µg/g in male mice.

Table 8. Neutralization of Mojave toxin in crude C. s. scutulatus venom.

Concentraion crude venom	Estimated conc. Mojave toxin	Concentration of monoclonal antibody 1	Deaths/Injected*
0.075 µg/g	0.163 nmol/ml	0.488 nmol/ml	0/4
0.150 µg/g	0.326 nmol/ml	0.976 nmol/ml	0/4
0.225 µg/g	0.489 nmol/ml	1.464 nmol/ml	0/4
0.300 µg/g	0.652 nmol/ml	1.952 nmol/ml	0/4

*24 hours. I. v. LD_{50} of crude C. s. scutulatus venom is 0.10 µg/g in male mice.

Table 9. Attempts to neutralize concolor toxin from C. v. concolor with monoclonal antibody 1.

Concentration toxin injected	Concolor toxin concentration	Concentration of monoclonal antibody 1	Deaths/Injected*
0.060 µg/g	0.261 nmol/ml	0.519 nmol/ml	0/4
0.100 µg/g	0.434 nmol/ml	0.868 nmol/ml	0/2
0.163 µg/g	0.706 nmol/ml	1.40 nmol/ml	2/3
0.225 µg/g	0.978 nmol/ml	1.95 nmol/ml	4/4

*24 hours. I. v. LD_{50} of purified concolor toxin is 0.075 µg/g in female mice.

Table 10. Experimentally determined and calculated molar extinction coefficients (E)* for selected toxins and subunits. ($E_{M,\ 280nm}$, pH 4-8)**

	Experimental¶	Calculated values
Intact Crotoxin	36,700+/- 320	42,000
Acidic subunit	8,530+/- 110	9,800
Bacic subunit	26,300+/- 280	32,200
Mojave toxin	39,000+/- 620	—

*A molecular weight of 23,340 Daltons was used for crotoxin and Mojave toxin; 8,840 Daltons for the acidic subunit; and 14,500 Daltons for the basic subunit.

**Intact crotoxin showed identical absorption intensity and wavelength maxima at both pH 4 and pH 7.8. Hanley (10) also reported absorbancies between pH 4 and 8 should be similar if not identical.

¶Samples were dried for 48-72 hrs under vaccum over P_2O_5. No corrections were made for water of hydration. Samples were weighed out on a Cahn 25 automatic electrobalance and dissolved in either 50mM Tris-HCl (pH 7.8) or 50 mM sodium acetate (pH4) at room temperature. All samples were spun in a microfuge before spectra were run in 1-cm silica cuvets in a HP 8450A spectrophotometer.

$E^{0.1\%=1\ mg/ml}$-values at 280nm (pH 4-8)	
Intact Crotoxin	1.57
Acidic subunit	0.964
Basic subunit	1.81
Mojave toxin	1.67

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